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(54) Title: TRANSGENIC ANIMAL MODELS FOR ALZHEIMER'S DISEASE

(57) Abstract

The construction of transgenic animal models for testing potential treatments for Alzheimer's disease are described. The models are characterized by a greater similarity to the conditions existing in naturally occurring Alzheimer's disease, based on expression of all three forms of the β-amyloid precursor protein (APP), APP_{695}, APP_{751}, and APP_{770}, as well as various point mutations based on naturally occurring mutations, such as the London and Indiana familial Alzheimer's disease (FAD) mutations at amino acid 717, and predicted mutations in the APP gene. The APP gene constructs are prepared using the naturally occurring promoter, as well as inducible promoters such as the mouse metallothionine promoter, which can be regulated by addition of heavy metals such as zinc to the animal's water or diet, and promoters such as the rat neuron specific enolase promoter, human β actin gene promoter, human platelet derived growth factor B (PDGF-B) chain gene promoter, rat sodium channel gene promoter, mouse myelin basic protein gene promoter, human copper-zinc superoxide dismutase gene promoter, and mammalian POU-domain regulatory gene promoter. The constructs are introduced into animal embryos using standard techniques such as microinjection. Animal cells can be isolated from the transgenic animals or prepared using the same contructs with standard techniques such as lipofection or electroporation. The transgenic animals, or animal cells, are used to screen for compounds altering the pathological course of Alzheimer's disease as measured by their effect on the amount and histopathology of APP and β-amyloid peptide in the animals, as well as by behavioral alterations.

Transgenic Animal Models for Alzheimer's Disease

Background of the Invention

Transgenic technology is described for the production of animals that exhibit symptoms of human Alzheimer's disease through the expression of the Alzheimer's precursor protein or a modified version thereof.

Alzheimer's Disease (AD) is a degenerative disorder of the brain first described by Alios Alzheimer in 1907 after examining one of his patients who suffered dramatic reduction in cognitive abilities and had generalized dementia ("The early story of Alzheimer's Disease", edited by Bick K., Amaducci L., and Pepeu G. (Raven Press, New York 1987). It is the leading cause of dementia in elderly persons. AD patients have increased problems with memory loss and intellectual functions which progress to the point where they cannot function as normal individuals. With the loss of intellectual skills the patients exhibit personality changes, socially inappropriate actions and schizophrenia ("A guide to the understanding of Alzheimer's Disease and related disorders", edited by Jorm AF.; (New York University Press, New York 1987). AD is devastating for both victims and their families, for there is no effective palliative or preventive treatment for the inevitable neurodegeneration. The most common problems in the Alzheimer's patient are inability to dress unaided, restlessness by day, urinary incontinence and sleep disturbances. The family members report embarrassment, anxiety, depression, and a decreased social life.

The impact of AD on society and on the national economy is enormous. It is expected that the demented elderly population in the United States will increase by 41% by the year 2000. It is expensive for the health care systems that must provide institutional and ancillary care for the patients at an estimated

neuropathy. However, available evidence strongly indicates two distinct types of genetic predisposition for AD. First, molecular analysis has provided evidence for mutations in the amyloid precursor protein (APP) gene in certain AD-stricken families (Goate, et al. Nature 349:704-706 (1991); Murrell, J, et al. Science 25 ; 97-99, 1991; Chartier-Harlin, M-C, et al. Nature 353 844-846 (1991)). Second, in certain other families with a clear genetic predisposition to AD, the mutation maps to chromosome 21 but is distinct from the APP locus (Tanzi, R.E., et al. Nature, 331;528-530 (1988)).

Amyloid plaques are abundantly present in AD patients and in Down's Syndrome individuals surviving to the age of 40. The plaques are also present in the normal aging brain, although at a lower number. These plaques are made up of the amyloid ß peptide (ß peptide) (Glenner and Wong, et al., Biochem. Biophys. Res. Comm. 120:885-890 (1984)), which is also the main protein constituent in cerebrovascular deposits and neurofibrillary tangles. The β peptide is a filamentous material that is arranged in beta-pleated sheets and has a molecular weight of 4.2-4.5 kd. It is a hydrophobic peptide comprising 39-42 amino acids. The determination of its amino acid sequence led to the cloning of the APP cDNA (Kang, et al., Nature 325:733-735 (1987); Goldgaber, et al., Science 235:877-880 (1987); Robakis et al., Proc. Natl. Acad. Sci. 84:4190-4194 (1987); Tanzi, et al., Nature 331:528-530 (1988) and genomic APP DNA (Lemaire et al., Nucl. Acids Res. 17:517-522 (1989); Yoshikai, et al., Gene 87, 257-263 (1990). Three forms of APP cDNAs (APP695, APP751, and APP770) have been isolated, and arise from a single precursor RNA by alternate splicing. The gene spans more than 175 Kb with 18 exons (Yoshikai, et al., 1990). APP contains three extracellular domains, a transmembrane region and a

of the full length protein (Goate, et al., (1991); Murrell et al., 1991; Chartier-Harlin et al., 1991). These mutations co-segregate with the disease within the families and are absent in families with late-onset AD.

There are no proven animal models to study AD, although aging nonhuman primates seem to develop amyloid plaques of β peptide in brain parenchyma and in the walls of some meningeal and cortical vessels. Although aged primates and canines can serve as animal models, they are expensive to maintain and need lengthy study periods. There are no spontaneous animal mutations with sufficient similarities to AD to be useful as experimental models. Various models have been proposed in which some AD-like symptoms may be induced by electrolysis, transplantation of AD brain samples, aluminum chloride, kainic acid or choline analogs (Kisner, et al., Neurobiol. Aging 7;287-292 (1986); Mistry, J. S., et al., J Med Chem 29;337-343 (1986)). Flood, et al. (Proc. Natl. Acad. Sci. 88:3363-3366 (1991)), reported amnestic effects in mice of four synthetic peptides homologous to the β peptide. Because none of these share with AD either common symptoms, biochemistry or pathogenesis, they are not likely to yield much useful information on etiology or treatment.

Transgenic mice with the human APP promoter linked to *E. coli* β-galactosidase (Wirak, D.O., et al., The EMBO J 10;289-296 (1991)) as well as transgenic mice expressing the human APP751 cDNA (Quon, D, et al. Nature 352, 239-241 (1991)) or subfragment of the cDNA including the β peptide (Wirak, D.O., et al., Science 253, 323-325 (1991); Sandhu, F.A., et al., J. Biol. Chem. 266, 21331-21334 (1991); Kawabata, S. Nature 354, 476-478 (1991)) have been produced. Results obtained in the different studies appear to depend upon the source of promoter

Alzheimer's disease is a complex syndrome involving pathological and behavioral aspects. A useful disease model should take these complexities into account. There are multiple proteins expressed from the gene with certain forms predominating in a given tissue. In the brain, the 695 form is predominant, but the mRNAs for additional forms are also present (Golde et al., <u>Neuron</u> 4; 253-267 (1990)). It is not known whether the ratio of the different forms changes with the age of the individual. The various protein forms result from alternative splicing such that the KI domain and/or the OX-2 domain may or may not be present in the mature protein. Moreover, the β-peptide results from post-translational processing of the precursor protein. This process can change in time as an individual ages, and can be affected by mutations not directly affecting the structure of the β-peptide:for example, the familial Alzheimer's disease (FAD) mutations at amino acid position 717 in the full length protein (Groate, et al., 1991; Murrell, et al., 1991; Chartier-Harlin, et al., 1991). Given these considerations, the production of universal animal models for Alzheimer's disease necessitates the construction of animal models that take into account the effects of known mutations on the phenotype resulting from the expression of these forms, and the possibility of the ratio of the different forms changing during the lifetime of the animal.

It is therefore an object of the present invention to provide an animal model for Alzheimer's disease that is constructed using transgenic technology.

It is a further object of the present invention to provide transgenic animals that accurately reflect the expression of different forms of the amyloid precursor protein

construct; the APP751 cDNA and bearing a mutation at amino acid 717; the APP695 cDNA; the APP695 cDNA bearing a mutation at amino acid 717; the APP leader sequence followed by the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP; the APP leader sequence followed by the ß peptide region plus the remaining carboxy terminal 56 amino acids with the addition of a mutation at amino acid 717; the APP leader sequence followed by the ß peptide region; the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP; the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP with the addition of a mutation at amino acid 717; a combination genomic-cDNA APP gene construct; and a combination genomic-cDNA APP gene construct, with the addition of a mutation at amino acid 717, operably linked to promoters selected from the following: the human APP gene promoter, mouse APP gene promoter, rat APP gene promoter, metallothionine gene promoter, rat neuron specific enolase gene promoter, human ß actin gene promoter, human platelet derived growth factor B (PDGF-B) chain gene promoter, rat sodium channel gene promoter, mouse myelin basic protein gene promoter, human copper-zinc superoxide dismutase gene promoter, and mammalian POU-domain regulatory gene promoter. Additional constructs include a human yeast artificial chromosome construct controlled by the human APP promoter; a human yeast artificial chromosome construct controlled by the human APP promoter with the addition of a mutation at amino acid 717; the endogenous mouse or rat APP gene modified through the process of homologous recombination between the APP gene in a mouse or rat embryonic stem (ES) cell and a vector carrying the human APP cDNA of the wild-type such that sequences in the resident rodent chromosomal APP gene beyond the recombination point (the preferred site for recombination is within APP exon 9) are

Figure 2b is a schematic of the APP751 cDNA coding sequence bearing a mutation at position 717.

Figure 3a is a schematic of the APP695 coding sequence.

Figure 3b is a schematic of the APP695 cDNA coding sequence bearing a mutation at position 717.

Figure 4a is a schematic of a coding sequence for the carboxy terminal portion of APP.

Figure 4b is a schematic of a coding sequence for the carboxy terminal portion of APP bearing a mutation at position 717.

Figure 5 is a schematic of a coding sequence for the ß peptide portion of APP.

Figure 6a is a schematic of a combination genomic/cDNA coding sequence allowing alternative splicing of the KI and OX2 exons.

Figure 6b is a schematic of a combination genomic/cDNA coding sequence bearing a mutation at position 717 and allowing alternative splicing of the KI and OX2 exons.

Figure 7a is a schematic of a human APP YAC coding sequence.

Figure 7b is a schematic of a human APP YAC coding sequence bearing a mutation at position 717.

Figure 8 is a schematic of genetic alteration of the mouse APP gene by homologous recombination between the mouse APP gene in a mouse ES cell and a vector carrying the human APP cDNA (either of the wild-type or FAD mutant form) directed to the exon 9 portion of the gene. As a result of this recombination event, sequences in the resident mouse chromosomal APP gene beyond the recombination point in exon 9 are replaced by the analogous human sequences.

Isolation of the uman APP promoter.

A cosmid li ary, constructed from human placental DNA in he pWE15 cosmid vector, was screened by hybridization ith a ^{32}P-labeled probe prepared by nick-translation (Maniatis, et al. Molecular Cloning: a laboratory man l (Cold Spring Harbor Laboratory, Cold Spring Harb , NY 1989)) of the APP770 cDNA clone. Clones t t hybridized with the probe were picked, purified, and characterized by restriction mapping, hybridi tion, and DNA sequencing. From one such clone conta ing a long 5′ flanking region, a *Not*I to *Nru*I rest iction DNA fragment of approximately 25 kb was isolat . This fragment terminates 2 nucleotides befo the initiator methionine codon of the Alzheimer's otein-coding region. This fragment, or a subfragment hereof, is the source of the human APP promoter for he constructs described herein. Analogous DNA fr ments isolated using the same methods from mou or rat genomic libraries are the source of mouse rat promoters.

Definition of AP cDNA clones.

The cDNA cl e APP-695 is of the form of cDNA described by Kan et al., Nature 325:733-735 ((1987), and represents t most predominant form of Alzheimer's prot n in the brain. The cDNA clone APP-751 is of the for described by Ponte, P, Nature 331, 525-527 (1988). he cDNA clone APP-770 is of the form described by Kit uchi, et al. Nature 331:530-532 (1988). This fo contains an insert of 225 nucleotides rela ve to the 695 form. The 225 nucleotide insert encodes for the KI domain as well as the OX-2 domain.

Definition of th APP genomic locus.

Characteriz ion of phage and cosmid clones of human genomic DN clones listed in the table below originally estab shed a minimum size of at least 100 kb for the Alzhe er's gene. There are a total of 18

addition to thos indicated above. First, an APP770 cDNA clone is di sted with Asp718 which cleaves after position 56 (num ring system of Kang et al., 1987). The resulting 5' xtension is filled in using the Klenow enzyme (M iatis et al., 1989) and ligated to a hexanucleotide o the following sequence: AGATCT, the recognition site or *Bgl*II. After cleavage with *Bgl*II, which als cuts after position 1769, and re-ligation, the tr slational reading frame of the protein is prese ed. The truncated protein thus encoded contains he leader sequence, followed by approximately 6 ino acids that precede the ß peptide, followe by the ß peptide, and the 56 terminal amino a ds of APP. The clone in Fig. 5 is created by the i roduction through site directed mutagenesis of n leotide 1913 in the clone of Fig. 4a (numbering syste of Kang et al., 1987) to a T thus creating a termi tion codon directly following the last amino acid c don of the β peptide. Each of the APP cDNA sequenc clones shown in Figs. 1-5 contains a single *Nru*I site nucleotides upstream from the initiator methio ne codon that is used for attachment of the different romoters used to complete each construct.

Expression ones identical to these but bearing mutations at the mino acid 717 of the full length protein, the sit of the FAD mutation, are also constructed. Mu tions at amino acid 717 are created by site-directed utagenesis (Vincent, et al., <u>Genes & Devel.</u> 3, 334-34 (1989)) and include mutations of the wild-type val co n to one of the following codons; ile, phe, gly, t , leu, ala, pro, trp, met, ser, thr, asn, gln.

The preferr method for construction of the combination cDNA/ enomic expression clones in Figure 6 is as follows. e *Taq*I site at position 860 (numbering syste of Kang, et al., 1987) in an APP770

Activity of Gene Promoters.

Different promoter sequences are used to control expression of APP coding sequences. The ability to regulate expression of the APP gene in transgenic animals is believed to be useful in evaluating the roles of the different APP gene products in AD. The ability to regulate expression of the APP gene in cultured cells is believed to be useful in evaluating expression and processing of the different APP gene products and may provide the basis for cell cultured drug screens.

The metallothionine (MT) promoter is well characterized, has been employed in transgenic animals, and its expression can be regulated through modulation of zinc and glucocorticoid hormone levels (Palmiter et al., Nature 300, 611-615 (1982)).

The human APP promoter is also characterized with regard to expression in the CNS (Wirak et al., 1991). It is believed that this promoter is useful for accurately reproducing temporal and spatial expression of human APP sequences in the CNS of transgenic rodents. In addition to the human APP promoter, the APP promoter from mouse and rat is used in conjunction with the various wild-type and mutant APP coding sequences. Although the human APP promoter has been shown to have activity in the appropriate regions of the brain of transgenic mice (Wirak et al., 1991), it is believed that the use of a mouse APP promoter in a transgenic mouse or a rat APP promoter in a transgenic rat will offer an even more precise pattern of expression in the CNS of transgenic animals.

As an alternative for the control of human APP expression in neurons, the rat neuron specific enolase gene promoter is used. This promoter has been shown to direct expression of coding sequences in neurons (Forss-Petter et al., Neuron 5;197-197 (1990)).

The YAC-APP lone, shown in Figu e 7a, is established in en ryonic stem (ES) ce ls by selecting for neomycin resi tance encoded by the YAC vector. ES cells bearing the YAC-APP clone are used to produce transgenic mice k established method described below under "Transgenic Mice" and "Embryoni Stem Cell Methods". The YA -APP gene bearing a mutation at amino acid 717 (F g. 7b) is produced through the generation of a Y C library using genomic DNA from a person affected k a mutation at amino acid 717. The clone is identifi d and established in ES cells as described above.

Genetic Alteratic of the Mouse APP G ne.

The nucleoti e sequence homology between the human and murine lzheimer's protein genes is approximately 85% Within the β pepti e-coding region, there are three amino acid differences between the two sequences The val residue that is mutated at amino acid 717 is conserved between mouse, rat, and man. Wild-type r dents do not develo Alzheimer's disease nor do th y develop deposits or plaques in their CNS analogo s to those present in human Alzheimer's patie ts. Therefore, it i possible that the human but not the rodent form of β peptide is capable of causir disease. Homologous recombination (Capecchi, MR Sci nce 244, 1288-1292 1989)) can be used to convert t e mouse Alzheimer's gene *in situ* to a gene encoding t e human ß peptide. This recombination is irected to a site downstream from the KI and OX-2 d mains, for example, within exon 9, so that the natur l alternative splicing mechanisms appropriate to al cells within the transgenic animal can be employed i expressing the final gene product.

Both wild-ty e (Fig. 8, schematic "a") and mutant (Fig. 8, schemati "b") forms of human cDNA are used to produce transg nic models expressing either the wild-type or muta t forms of APP. The recombination

is redissolved in ml of low salt bu fe (0.2 M NaCl, 20 mM Tris™, pH 7 4, and 1 mM EDTA) a d urified on an Elutip-D™ column. The column is firs imed with 3 ml of high salt b fer (1 M NaCl, 20 m ris™, pH 7.4, and 1 mM EDTA) fo owed by washing w th ml of low salt buffer. The NA solutions are ss l through the column for three mes to bind DNA t h column matrix. After on wash with 3 ml of w alt buffer, the DNA is eluted ith 0.4 ml of high buffer and precipitated by t volumes of ethan NA concentrations ar measured by absor t o at 260 nm in a UV spectrophoto ter. For microin cti n, DNA concentrations ar adjusted to 3 μg/ i 5 mM Tris™, pH 7.4 and 0.1 mM DTA. Other method f purification of D for microinjectio also described in Hoga et al., <u>Manipula he mouse embryo</u> (cold Spri Harbor Laborato d Spring Harbor, NY (1986), in Palmiter, et a , <u>ture</u> 300, 611 (1982), in "T Qiagenologist, A li tion Protocols", 3rd e tion, published b i en, Inc., Chatsworth, CA., d in Maniatis, et . <u>Molecular Cloning: a labora ry manual</u> (Cold S Harbor Laboratory, Cold ring Harbor, NY 1

<u>Construction of T nsgenic Animals</u>.

<u>Animal Sourc</u>

Animals suit le for transgenic p iments can be obtained from andard commercial u es such as Charles River (Wi ington, MA), Tac ermantown, NY), Harlan Sprag Dawley (Indiana IN), etc. Swiss Webster fem e mice are prefe r embryo retrieval and tra fer. B6D2F$_1$ males n e used for mating and vasect ized Swiss Webste t s can be used to stimulate seudopregnancy. e omized mice and rats can be o ained from the su .

<u>Microinjecti Procedures</u>

The procedur for manipulatio e rodent embryo and for mic oinjection of DNA e escribed in

transferred. After the transfer, the incision is closed by two sutures.

Transgenic Rats

The procedure for generating transgenic rats is similar to that of mice (Hammer et al., Cell 63;1099-112 (1990)). Thirty day-old female rats are given a subcutaneous injection of 20 IU of PMSG (0.1 cc) and 48 hours later each female placed with a proven male. At the same time, 40-80 day old females are placed in cages with vasectomized males. These will provide the foster mothers for embryo transfer. The next morning females are checked for vaginal plugs. Females who have mated with vasectomized males are held aside until the time of transfer. Donor females that have mated are sacrificed (CO_2 asphyxiation) and their oviducts removed, placed in DPBS (Dulbecco's phosphate buffered saline) with 0.5% BSA and the embryos collected. Cumulus cells surrounding the embryos are removed with hyaluronidase (1 mg/ml). The embryos are then washed and placed in EBSS (Earle's balanced salt solution) containing 0.5% BSA in a [illegible] °C incubator until the time of microinjection.

Once the embryos are injected, the live embryos are moved to DPBS for transfer into foster mothers. The foster mothers are anesthetized with ketamine (40 mg/kg, ip) and xylazine (5 mg/kg, ip). A dorsal midline incision is made through the skin and the ovary and oviduct are exposed by an incision through the muscle layer directly over the ovary. The ovarian bursa is torn, the embryos are picked up into the transfer pipet, and the tip of the transfer pipet is inserted into the infundibulum. Approximately 10-12 embryos are transferred into each rat oviduct through the infundibulum. The incision is then closed with sutures, and the foster mothers are housed singly.

DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination, described by Capecchi, (1989). Direct injection results in a high efficiency of integration. Desired clones are identified through PCR of DNA prepared from pools of injected ES cells. Positive cells within the pools are identified by PCR subsequent to cell cloning (Zimmer and Gruss, Nature 338, 150-153 (1989)). DNA introduction by electroporation is less efficient and requires a selection step. Methods for positive selection of the recombination event (i.e., neo resistance) and dual positive-negative selection (i.e., neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Joyner et al., Nature 338, 153-156 (1989) and Capecchi, (1989), the teachings of which are incorporated herein.

Embryo Recovery and ES cell Injection

Naturally cycling or superovulated female mice mated with males are used to harvest embryos for the implantation of ES cells. It is desirable to use the C57B strain for this purpose when using mice. Embryos of the appropriate age are recovered approximately 3.5 days after successful mating. Mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are flushed from excised uterine horns and placed in Dulbecco's modified essential medium plus 10% calf serum for injection with ES cells. Approximately 10-20 ES cells are injected into blastocysts using a glass microneedle with an internal diameter of approximately 20 µm.

Transfer of Embryos to Pseudopregnant Females

Randomly cycling adult female mice are paired with vasectomized males. Mouse strains such as Swiss Webster, ICR or others can be used for this purpose.

observed in deeper grey matter including the amygdaloid nucleus corpus striatum and diencephalon. Sections are also stained with other antibodies diagnostic of Alzheimer's plaques, recognizing antigens such as Alz-50, tau, A2B5, neurofilaments, neuron-specific enolase, and others that are characteristic of Alzheimer's plaques (Wozin, et al., Science 232, 648 (1986); Hardy and Allsop, Trends in Pharm. Sci. 12, 383-388 (1991); Selkoe, Ann. Rev. Neurosci. 12, 463-490 (1989); Arai et al., Proc. Natl. Acad. Sci. USA 87, 2249-2253 (1990); Majocha et al., Amer. Assoc. Neuropathology Abs; 99,22 (1988); Masters et al., Proc. Natl Acad. Sci. 82,4245-4249; Majocha et al., Can J Biochem Cell Biol 63;577-5 (1985)). Staining with thioflavins and congo red is also carried out to analyze co-localization of ß peptide deposits within neuritic plaques and NFTs.

Analysis of APP and ß Peptide Expression:

mRNA: mRNA is isolated by the acid guanidinium thiocyanate-phenol chloroform extraction method (Chomczynski and Sacchi, Anal Biochem 16,156-159 (1987)) from cell lines and tissues of transgenic animals to determine expression levels by Northern blots.

Protein: APP and ß peptide are detected by using polyclonal and monoclonal antibodies that are specific to the extra-cytoplasmic domain, ß peptide region, and C-terminus.

Western Blot Analysis: Protein fractions are isolated from tissue homogenates and cell lysates and subjected to Western blot analysis as described by Harlow et al., Antibodies: A laboratory Manual, (Cold Spring Harbor, NY, 1988); Brown et al., J. Neurochem. 40;299-308 (1983); and Tate-Ostroff et al., Proc Natl Acad Sci 86;745-74 (1989)). Only a brief description is given below.

are detected by autoradiography or enzym ... lly labeled probes are detected through reac ... n with the appropriate chromogenic substrates.

Behavioral Studies

Behavioral tests designed to assess le ... ning and memory deficits are employed. An exampl ... such as test is the Morris Water maze (Morris, L ... Motivat. 12;239-260 (1981)). In this procedure, ... animal is placed in a circular pool filled with wa ... with an escape platform submerged just below the ... ace of the water. A visible marker is placed o ... platform so that the animal can find it by naviga ... toward a proximal visual cue. Alternatively, a m ... omplex form of the test in which there are no f ... cues to mark the platform's location will be gi ... r the animals. In this form, the animal must ... the platform's location relative to distal v ... cues.

The procedures applied to test tran ... c mice is similar for transgenic rats.

Screening of Compounds for Treatment of ... imer's Disease

The transgenic animals and animal c ... re used to screen compounds for a potential effe ... the treatment of Alzheimer's disease using s r ... d methodology. The compound is administer ... the animals or introduced into the culture m ... over a period of time and in various dosages, t ... e. animals or animal cells examined for alt ... ns in APP expression, histopathology, and/or b ... r using the procedures described above.

Example 1: Expression of pMTAPP-1 ir ... 3 and PC12 Cells.

The clone, pMTAPP-1 is an example o ... expression vector shown in Fig. 1a where ... romoter used is the metallothionine promoter. S ... cell lines were derived by transfecting NIH3T ... PC12 cell lines (ATCC #CCL92 and CRL1721). 0. ... ^{6} of

human APP770 cDNA like the construct in ure 1A. DNA from this construct was transfected PC12 cells as described above. Certain clones EAPP-1 transfected cells exhibited a differenti n phenotype morphologically similar to tha xhibited by PC12 cells treated with nerve growth fac (NGF). PC12 cells normally are fairly round and t cells. Those transfected with pEAPP-1 have cyto mic extensions resembling neurites. PC12 cel treated with NGF extend very long neuritic extens s. Thirteen PC12 cell clones transfected wi EAPP-1 were selected and propagated. Eight of e cell clones exhibited the spontaneous differe tion phenotype with clones 1-8, 1-1, and 1-4 biting the strongest phenotypes. Staining of pEAPP ransfected PC12 cells with antibody against the ß pe de as described above indicated that those cel xhibiting the differentiation were also expressing . Because PC12 cells transfected with the pMTAPP1 e did not exhibit this phenotype even though the A 0 cDNA is expressed, these results suggest tha ex sion of APP770 from the human promoter has novel erties regarding the physiology of the cell.

Example 3: **Expression of pMTA4 in PC ells.**

pMTA4 is an example of the type of truct shown in Figure 4A where the promoter use s the metallothionine promoter. The protein er ed by this construct differs slightly from tha dep d in Figure 4. An APP770 cDNA clone was ges with Asp718 which cleaves after position 6 (r system of Kang, et al., 1987). The resulting 5 tension was filled in using the Klenow enzyme (Ma tis). The same DNA preparation was also cleaved wit coRI which also cuts after position 1795 and th res ing 5' extension was filled in using the Klenow e (Maniatis). Self-ligation of this molec results in an expression clone in which the truncat otein

a humidified atmosphere at 7% CO_2, 5% O_2, and 88% N_2 until the time of injection.

Microinjection: Elutip-D™ purified [illegible] DNA was dissolved in 5 mM Tris (pH 7.4) and 0.1 [illegible] TA at 3 μg/ml concentration for microinjection. [illegible]oneedles and holding pipettes were pulled from Fi[illegible] coagulation tubes (Fisher) on a DKI mode 72[illegible] pipette puller. Holding pipettes were then broken at approximately 70 μm (O.D.) and fire polished to an I.D. of about 30 μm on a Narishige micro[illegible] (model MF-83). Pipettes were mounted on Narishig micromanipulators which were attached to [illegible]ikon Diaphot microscope. The air-filled injection pipette was filled with DNA solution through the tip after breaking the tip against the holding pipette. Embryos, in groups of 30 to 40, were pla[illegible] in 100 μl drops of EBBS under paraffin oil for micromanipulation. An embryo was oriented and held with the holding pipette. The injection pipette was then inserted into the male pronucleus [illegible]sua[illegible]y the larger one). If the pipette did not bre[illegible] through the membrane immediately the stage was tapp[illegible] to assist in penetration. The nucleus was then injected and the injection was monitored by swelling of the nucleus. Following injection, the group of embry[illegible]s placed in EBSS until transfer to recipient fe[illegible]es.

Transfer: Randomly cycling adult [illegible]al[illegible] mice were paired with vasectomized Swiss Web[illegible]r [illegible]ales. Recipient females were mated at the sam[illegible] as donor females. At the time of transfer, the [illegible] were anesthetized with avertin. The oviduct [illegible] exposed by a single midline dorsal incision. [illegible] ision was then made through the body wall directl[illegible] the oviduct. The ovarian bursa was then to[illegible] with watch makers forceps. Embryos to be transf[illegible] re placed in DPBS and in the tip of a transfer pi[illegible] bout 10-12 embryos). The pipet tip was inserted [illegible] the

We claim:

1. A non-human transgenic mammal o[illegible]ammalian cells containing a construct selected fr[illegible] group consisting of the APP770 cDNA; the APP77[illegible]NA bearing a mutation at amino acid 717; the APP751 [illegible]A containing the KI protease inhibitor doma[illegible]ithout the OX2 domain in the construct; the APP7[illegible] cDNA bearing a mutation at amino acid 717; th[illegible]P695 cDNA; the APP695 cDNA bearing a mutation at am[illegible]cid 717; the APP leader sequence followed by the [illegible]ptide region plus the remaining carboxy termin[illegible]6 amino acids of APP; the APP leader sequence fo[illegible]ed by the ß peptide region plus the remaining carbo[illegible] terminal 56 amino acids with the addition of a mut[illegible]n at amino acid 717; APP leader sequence foll[illegible] by the ß peptide region; the ß peptide region plu[illegible] remaining carboxy terminal 56 amino acid[illegible]PP; the ß peptide region plus the remaining carb[illegible] terminal 56 amino acids of APP with the addition [illegible]utation at amino acid 717; a combination genomic-[illegible] APP gene construct; a combination genomic-cDNA APP [illegible]e construct, with the addition of a mutati[illegible] amino acid 717, operably linked to a promoter [illegible]ed from the following: the human APP promoter, [illegible]PP promoter, rat APP promoter, metallothion[illegible] promoter, rat neuron specific enolase promoter, hu[illegible] actin gene promoter, human platelet derived gr[illegible]factor B (PDGF-B) chain gene promoter, rat sodium [illegible]nel gene promoter, mouse myelin basic protein gen[illegible]moter, human copper-zinc superoxide dismutase [illegible]romoter, and mammalian POU-domain regulatory gen[illegible] ter; and combinations thereof.

2. The transgenic mammal of claim 1 [illegible]essing any of the constructs

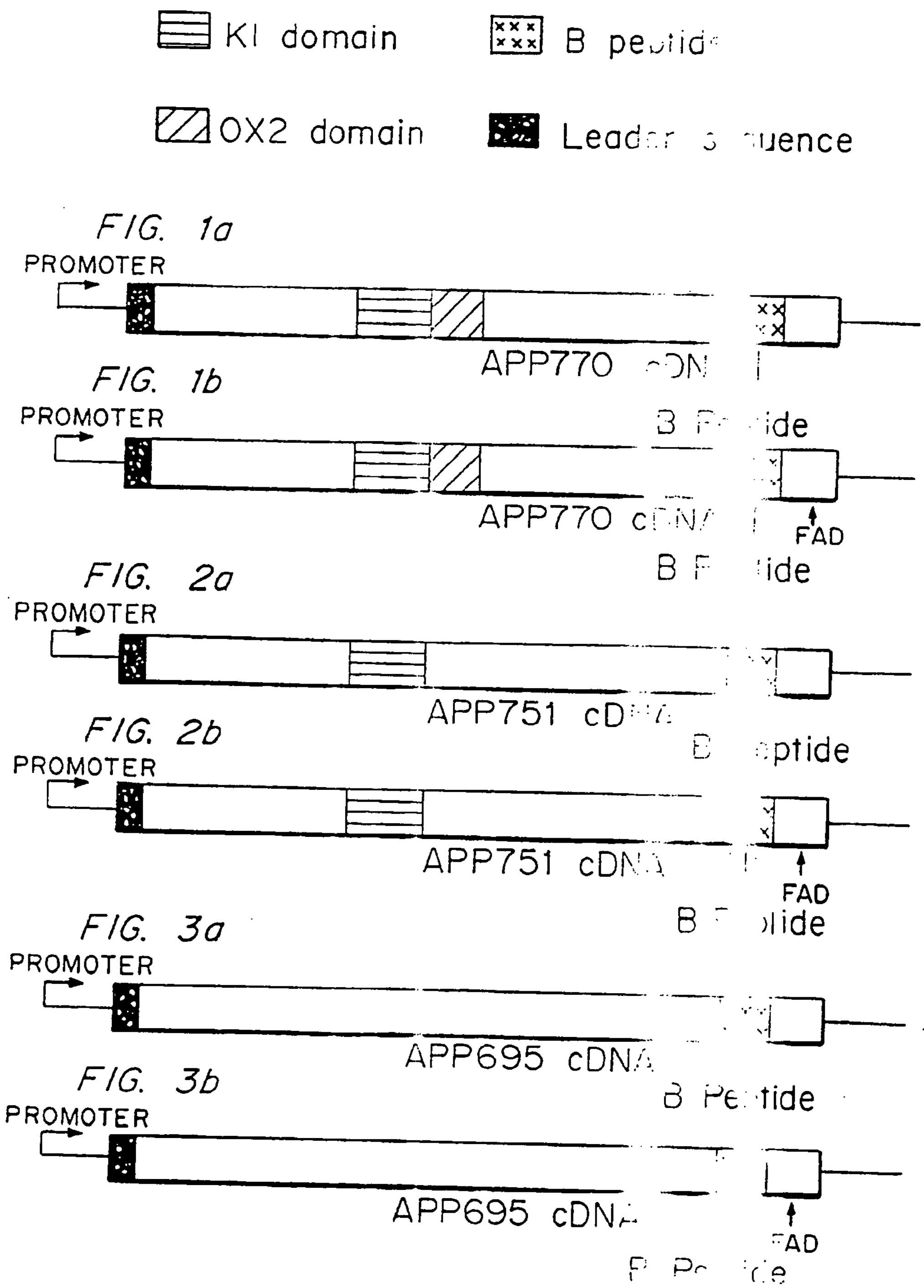
3. Cells cultured from the transge[illegible]ammal of claim 1.

combination genomic-cDNA APP gene construct, with the addition of a mutation at amino acid 717, operably linked to a promoter selected from the following: the human APP promoter, mouse APP promoter, rat APP promoter, metallothionine promoter, rat neuron specific enolase promoter, human ß actin gene promoter, human platelet derived growth factor B (PDGF-B) chain gene promoter, rat sodium channel gene promoter, mouse myelin basic protein gene promoter, human copper-zinc superoxide dismutase gene promoter, and mammalian POU-domain regulatory gene promoter; and combinations thereof.

10. The method of claim 9 wherein the transgenic model is a mammal having altered behavior.

11. A method for screening compounds for an effect on Alzheimer's disease comprising

exposing a transgenic mammal or mammalian cells containing a construct selected from the group consisting of the APP770 cDNA; the APP770 cDNA bearing a mutation at amino acid 717; the APP751 cDNA containing the KI protease inhibitor domain without the OX2 domain in the construct; the APP751 cDNA bearing a mutation at amino acid 717; the APP695 cDNA; the APP695 cDNA bearing a mutation at amino acid 717; the APP leader sequence followed by the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP; the APP leader sequence followed by the ß peptide region plus the remaining carboxy terminal 56 amino acids with the addition of a mutation at amino acid 717; APP leader sequence followed by the ß peptide region; the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP; the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP with the addition of a mutation at amino acid 717; a combination genomic-cDNA APP gene construct; a combination genomic-cDNA APP gene construct, with the addition of a mutation at amino


KI domain
B peptide
OX2 domain
Leader sequence
FIG. 1a
PROMOTER
APP770 cDNA
FIG. 1b
PROMOTER
B Peptide
APP770 cDNA
FAD
B Peptide
FIG. 2a
PROMOTER
APP751 cDNA
FIG. 2b
PROMOTER
B Peptide
APP751 cDNA
FAD
B Peptide
FIG. 3a
PROMOTER
APP695 cDNA
FIG. 3b
PROMOTER
B Peptide
APP695 cDNA
FAD
B Peptide

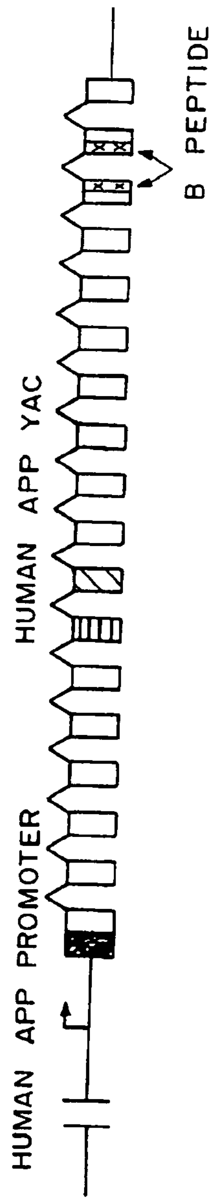


FIG. 7a

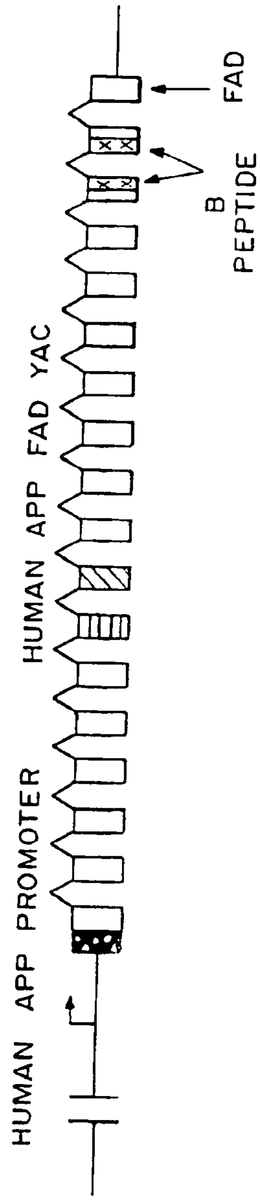


FIG. 7b

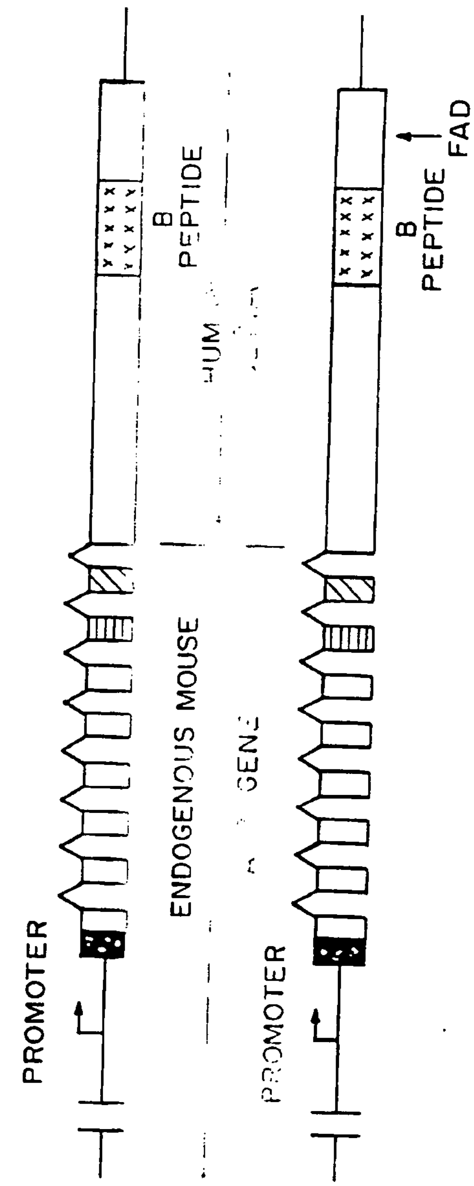


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/11276

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Current opinion in neurology and neurosurgery, Volume 5, 1992, B. T. Hyman et al., "Amyloid, dementia and Alzheimer's disease", page 88 - page 93, fig 2, page 88, right column - page 89, left column, page 90, right column	1-13
Y		1-13
	--	
X	Journal of Biological Chemistry, Volume 266, No 32, November 1991, F. A. Sandhu et al., "Expression of the Human beta-Amyloid Protein of Alzheimer's Disease Specifically in the Brains of Transgenic Mice", pages 21331-21334, Fig 1, page 21334	1-6,9,10
Y		1-13
	--	
X	WO, A1, 8906689 (THE MCLEAN HOSPITAL CORPORATION), 27 July 1989 (27.07.89), page 21, line 18 - line 20; page 40 - page 46, figure 8, page 40, last paragraph	1,9
	--	
X	Nature, Volume 354, December 1991, S. Kawabata et al., "Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein", page 476 - page 478, figure 1, page 478	1-6,9-13
	--	
X	EP, A1, 0451700 (D. O. WIRAK), 16 October 1991 (16.10.91), page 6, line 35 - page 7, line 5; page 10 - page 13, examples 11-16	1-6,9-13
	--	
X	Science, Volume 253, July 1991, D. O. Wirak et al., "Deposits of Amyloid beta Protein in the Central Nervous System of Transgenic Mice", page 323 - page 325, page 323	1,9
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/11276

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The present application lacks unity of invention a posteriori - see the cited documents. Thus, each of the claimed solutions in claims 1, 9 and 11 to the known problem of creating a transgenic animal modell for Alzheimers disease, represents a separate inventive concept.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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FI	Finland				

annual cost of $40 billion (Jorm, 1987; Fisher, LM: New York Times, August 23, 1989 D1 "Alzheimer's Disease", edited by Reisberg, B.; (The Free Press, New York & London 1983). These factors imply preventive action must be taken to decrease AD incidence by allocating resources into AD research.

At a macroscopic level, the brains of AD patients are usually smaller, sometimes weighing less than 1,000 grams. At a microscopic level, the histopathological symptoms of AD include neurofibrillary tangles (NFT), neuritic plaques, and degeneration of neurons. AD patients exhibit degeneration of nerve cells in the frontal and temporal cortex of the cerebral cortex, pyramidal neurons of hippocampus, neurons in the medial, medial central, and cortical nuclei of the amygdala, noradrenergic neurons in the locus coeruleus, and the neurons in the basal forebrain cholinergic system. Loss of neurons in the cholinergic system leads to a consistent deficit in cholinergic presynaptic markers in AD (Reisberg, 1983; "Alzheimer's Disease and related disorders, research and development" edited by Kelly WE; (Charles C. Thomas, Springfield, IL. 1984).

AD is associated with neuritic plaques measuring up to 200 μm in diameter in the cortex, hippocampus, subiculum, hippocampal gyrus, and amygdala. One of the principal constituents of neuritic plaques is amyloid, which is stained by congo red (Reisberg, 1983; Kelly, 1984). Amyloid plaques are extracellular, pink - or rust-colored in bright field, and birefringent in polarized light. The plaques are composed of polypeptide fibrils and are often present around blood vessels, reducing blood supply to various neurons in the brain.

Various factors such as genetic predisposition, infectious agents, toxins, metals, and head trauma have all been suggested as possible mechanisms of AD

cytoplasmic domain. The ß peptide consists of 28 amino acids just outside the membrane and 14 residues of the hydrophobic transmembrane domain. Thus, the ß peptide is a cleavage product of APP normally found in brain and other tissues such as heart, kidney and spleen. ß peptide deposits, however, are usually found only in the brain, although Joachim et al., <u>Nature</u> 341:226-228 (1989) have reported ß peptide deposits outside the brain in the skin, intestine, and subcutaneous tissues of most AD patients.

The larger alternate forms of APP (APP751, APP770) consist of all of APP695 plus one or two additional domains. APP751 consists of all of APP695 plus an additional 56 amino acids which has homology to the Kunitz family of serine protease inhibitors (KPI) (Tanzi et al., 1988; Weidemann et al., <u>Cell</u> 57:115-126 (1989); Kitaguchi, et al. <u>Nature</u> 331:530-532 (1988); Tanzi et al., <u>Nature</u> 329:156 (1987). APP770 contains APP751 and an additional 19 amino acid domain homologous to the neuron cell surface antigen OX-2 (Weidemann, et al., <u>Cell</u> 57:115-126 (1989); Kitaguchi et al., 1988). APP is post-translationally modified by the removal of the leader sequence and by the addition of sulfate and sugar groups.

Van Broeckhaven, et al., <u>Science</u> 248:1120-1122 (1990) have demonstrated that the APP gene is tightly linked to hereditary cerebral hemorrhage with amyloidosis (HCHWA-D) in two Dutch families. This was confirmed by the finding of a point mutation in the APP coding region in two Dutch patients (Levy et al., <u>Science</u> 248:1124-1128 (1990). The mutation substituted a glutamine for glutamic acid at position 22 of the ß peptide (position 618 of APP695). In addition, certain families are genetically predisposed to Alzheimer's disease, a condition referred to as familial Alzheimer's disease (FAD), through mutations resulting in an amino acid replacement at position 717

and the protein coding sequence use For example, Wirak, et al. (1991) found that in ansgenic mice expressing a form of the ß peptide, ntracellular deposits of "amyloid-like" material reactive with antibodies prepared against APP wer observed but did not find other histopathological di ase symptoms. The intracellular nature of the ant ody-reactive material and the lack of other symp ms suggest that this particular transgenic animal i not a faithful model system for Alzheimer's diseas Kawabata et al. (1991) report the production of amy id plaques, neurofibrillary tangles, and neuron cell death in their transgenic animals. In each these studies, the same peptide fragment, the ß pe ide plus the 56 remaining C terminal amino acids of PP, was expressed. Wirak et al. (1991) use the human APP promoter while Kawabata, et al. (19) used the human thy-1 promoter. In transgenic mice xpressing the APP751 cDNA from the neuron-specifi enolase promoter of Quon, D., et al., Nature 352, 23 241 (1991), extracellular deposits of material active with antibody prepared against APP were served. What was not shown was whether the deposits ntained full-length APP751 or ß peptide or both, hus precluding any correlation of the deposits wit those present in Alzheimer's disease. Quon et al. (91) also state that the protein encoded by the APP 5 cDNA expressed from the neuron-specific enolase pr oter, does not form extracellular immunoreactive d osits. These results raise the possibility that though the ß pept_de is included within the APP6 precursor, use of the neuron-specific enolase prom er in conjunction with the APP695 cDNA may not presen an effective Alzheimer's disease model. Further re, the presence of APP immunoreactive deposits is n correlated with the age or gene dosage in their par cular transgenic model.

It is a still further object o the present invention to provide transgenic ani ls characterized by certain genetic abnormalities in he expression of the amyloid precursor protein.

Summary of the Inve on

The construction of transgenic nimal models for testing potential treatments for Al eimer's disease is described. The models are chara erized by a greater similarity to the condition existing in naturally occurring Alzheimer's dis se, based on the ability to control expression of on or more of the three forms of the ß-amyloid precur r protein (APP), APP695, APP751, and APP770, or subf gments thereof, as well as various point mutations sed on naturally occurring mutations, such as the FA mutations at amino acid 717, and predicted mutat ns in the APP gene. The APP gene constructs are epared using the naturally occurring APP promoter of uman, mouse, or rat origin, as well as inducible pr oters such as the mouse metallothionine promoter, whi . can be regulated by addition of heavy metals such as inc to the animal's water or diet. Neuron-spe fic expression of constructs is achieved by using the at neuron specific enolase promoter.

The constructs are introduced to animal embryos using standard techniques such as m roinjection or embryonic stem cells. Cell culture ased models can also be prepared by two methods. C l cultures can be isolated from the transgenic animal or prepared from established cell cultures using the ame constructs with standard cell transfection tec iques.

The specific constructs that a e described employ the following protein coding sequen s: the APP770 cDNA; the APP770 CDNA bearing a mut ion at amino acid 717; the APP751 cDNA containing th KI protease inhibitor domain without the OX2 do ain in the

replaced by the analogous human seq nces; the endogenous mouse or rat APP gene mo fied through the process of homologous recombination etween the APP gene in a mouse or rat ES cell and vector carrying the human APP cDNA bearing a mutati at amino acid position 717 such that sequences in he resident rodent chromosomal APP gene beyond e recombination point (the preferred site for recom nation is within APP exon 9) are replaced by the ana gous human sequences bearing a mutation at ami acid 717. These constructs can be introduced into t transgenic animals and then combined by mating f animals expressing the different constructs

The transgenic animals, or ani l cells, are used to screen for compounds altering th pathological course of Alzheimer's Disease as me ured by their effect on the amount and histopatho gy of APP and ß peptide in the animals, as well as behavioral alterations.

Brief Description of the rawings

The boxed portions of the draw gs indicate the amino acid coding portions of the c structs. Filled portions indicate the various domai of the protein as indicated in the Figure Legend. ines indicate sequences in the clones that are 5' r 3' untranslated sequences, flanking genomic sequenc , or introns. The break in the line to the left o the constructs in Figs. 7 and 8 indicates the presenc of a long DNA sequence.

Figure 1a is a schematic of th APP 770 cDNA coding sequence.

Figure 1b is a schematic of th APP770 cDNA coding sequence bearing a mutation position 717.

Figure 2a is a schematic of th APP751 cDNA coding sequence.

Detailed Description of th Invention

The constructs and transgenic imals and animal cells are prepared using the method ind materials described below.

Sources of materials.

Restriction endonucleases are tained from conventional commercial sources suc as New England Biolabs (Beverly, MA.), Promega Bio gical Research Products (Madison, WI.), and Strata ne (LaJolla CA.), etc. Radioactive materials are obt ned from conventional commercial sources suc as Dupont/NEN or Amersham. Custom-designed oligonuc otides for site-directed mutagenesis are available om any of several commercial providers of such materi s such as Bio-Synthesis Inc., Lewisville, TX. Ki for carrying out site-directed mutagenesis are avail le from commercial suppliers such as Promeg Biological Research Products, Stratagene, etc. Clones of cDNA including the APP695, APP751, and A 770 forms of APP mRNA were obtained directly from Dr Dmitry Goldgaber, NIH. Libraries of DNA are availabl from commercial providers such as Stratagene, La Jo a, CA., or Clontech, Palo Alto, CA. PC12 and 3 cells were obtained from ATCC (#CRL1721 and #C 92 respectively). An additional PC12 cell line was ob ined from Dr. Charles Marotta of Harvard Medical hool, Massachusetts General Hospital, and cLean Hospital. Standard cell culture media appropr te to the cell line are obtained from conventional ommercial sources such as Gibco/BRL. Murine stem cel , strain D3, were obtained from Dr. Rolf Kemler (Doet hman, et al., J. Embryol. Exp. Morphol. 87, 27 (1985 . Lipofectin for DNA transfection and the drug G418 or selection of stable transformants are available om Gibco/BRL.

exons in the APP gene (Lemaire et a ., Nucl. Acid Res, 17;517-522, 1989; Yoshikai et al., 90). These results taken together indicate tha the minimum size of the Alzheimer's gene is 175 kb.

I. Table of Alzheimer's Cosm and Lambda Clones

Library	Name of Clone	Insert Size (Kb)	signed APP Region
1 Cosmid 1	1 GPAPP47A	35	5 Kb promoter & 9 Kb intron
	2 GPAAP36A	35	2 Kb promoter & 22 Kb intron
	3 GAPP30A	30-35	' coding region
	4GAPP43A	30-35	xons 9, 10 and 11
Lambda	1 GAPP6A	12	xon 6
	2 GAPP6B	18	xons 4 and 5
	3 GAPP20A	20	xon 6
	4 GAPP20B	17	xons 4 and 5
	5 GAPP28A	18	xons 4 and 5
	6 GAPP3A	14	xon 6
	7 GAPP4A	19	xon 6
	8 GAPP10A	16	xons 9, 10 and 11
	9 GAPP16A	21	xon 6

Construction of Transgenes.

The clones bearing various po ions of the human APP gene sequence shown in Figs. 1- are constructed in an analogous manner. First, th polyA addition signal from SV40 virus as a 253 ba pair *BclI* to *BamHI* fragment (Reddy et al., Scie 200;494-502 (1978) is cloned into a modified v tor from the pUC series. Next, the cDNA coding seq nces (770, 751, or 695) are inserted. Correct orient ion and content of the fragments inserted is determin through restriction endonuclease mapping a limited sequencing.

The clones bearing various ca oxy terminal portions of the human APP gene seq nce shown in Figs. 4 and 5 are constructed through se ral steps in

cDNA clone is converted to an *Xho*I s e by site-directed mutagenesis. Cleavage of t resulting plasmid with *Xho*I cuts at the new *Xh* site and a pre-existing site at 930, and releases t KI and OX-2 coding sequence.

The plasmid thus generated serv as the acceptor for the KI and OX-2 alternative spli ng cassette. The alternative splicing cassette is reated through a series of cloning steps. First, the aqI site at position 860 (numbering system of Ka , et al., 1987) in the genomic clone containing exor and adjacent downstream intron is converted to ar hoI site by site-directed mutagenesis. Cleavage f the resulting plasmid with *Xho*I cuts at the new *Xh* site and an *Xho*I site within the adjacent intror This fragment is cloned into the *Xho*I site in a pl mid vector. Second, the genomic clone containing xon 9 and adjacent upstream intron is cleaved ith *Xho*I (position 930) and cloned into the X I site of a plasmid vector. These two junction on/intron fragments are released from their re pective plasmid backbones by cleavage with *Xho*I and ither *Bam*HI or *Bgl*II, and cloned into the *Xho*I site of a plasmid vector. The resulting *Xho*I fragment is cleaved with either *Bam*HI or *Bgl*II and the genom 6.6 kb *Bam*HI segment (Kitaguchi et al., 1988) co aining the KI and OX-2 coding region along with their lanking intron sequences are inserted. After clea ge with *Xho*I, this DNA segment is inserted into t *Xho*I site of the modified APP770 cDNA constructed ab e. These cloning steps generate a combination cDNA/g omic expression clone that allows cells in a transg ic animal to regulate the inclusion of the KI an OX-2 domains by a natural alternative splicing mechan m. An analogous gene bearing a mutation at amino ac 717 is constructed by using the mutated fo of APP770 cDNA described above.

Other alternatives for use in c trolling human APP expression in neurons include th human ß actin gene promoter (Ray et al., Genes and evelopment 5:2265-2273 (1991)), the human plate t derived growth factor B (PDGF-B) chain gene promote (Sasahara et al., Cell 64:217-227 (1991)), the ra sodium channel gene promoter (Maue et al., Neuron 4 23-231 (1990)), the human copper-zinc superoxide dis tase gene promoter (Ceballos-Picot et al., Bra Res. 552:198-214 (1991)), and promoters for membe of the mammalian POU-domain regulatory gene amily (Xi et al., Nature 340:35-42 (1989)). The U-domain is the region of similarity between the fou mammalian transcription factors Pit-1, Oct-1, t-2, and *unc*-86, and represents a portion of the DNA- inding domain. These promoters are known or believe to result in expression specifically within the rons of transgenic animals.

Expression of human APP in non- uronal brain cells can be directed by the promot for mouse myelin basic protein (Readhead et al., Cel 8:703-712 (1987)).

Yeast Artificial Chromosomes.

The constructs shown in Figure are constructed as follows. Large segments of huma genomic DNA, when cloned into certain vectors, can be ropagated as autonomously-replicating units in t yeast cell. Such vector-borne segments are refe ed to as yeast artificial chromosomes (YAC; Burke al. Science 236, 806 (1987)). A human YAC library i commercially available (Clontech, Palo Alto, CA) ith an average insert size of 250,000 base pairs (nge of 180,000 to 500,000 base pairs). A YAC clone o the Alzheimer's gene can be directly isolated by sc ening the library with the human APP770 cDNA. The in usion of all of the essential gene regions in the c ne can be confirmed by PCR analysis.

vector is constructed from a human P cDNA (695 or 770 form), either wild-type or muta . at amino acid 717. Cleavage of the recombinatio ector, for example, at the *Xho*I site within e . 9, promotes homologous recombination within the irectly adjacent sequences (Capecchi, 1989). The e genous APP gene resulting from this event is norma p to the point of recombination, within exon 9 in th example, and consists of the human cDNA sequenc hereafter.

Mutant Forms of APP Proteins

Expression clones identical t hese but bearing mutations at the amino acid 717 of e full length protein, the site of FAD mutations, re also constructed. Mutations at amino a 717 are created by site-directed mutagenesis (Vinc , et al., 1989) and include mutations of the wild-t e val codon to one of the following codons; ile, , gly, tyr, leu, ala, pro, trp, met, ser, thr, asn, n. Mutations of val-717 to ile, phe, and gly, have en described (Goate et al., 1991; Murrell, et a , 1991; Chartier-harlin et al., 1991). None of the naturally-occurring mutations are charged or lky amino acids. Therefore it is believed that repl ment of val-717 with the other amino acids listed also promote the FAD syndrome and have properties t are useful for animal AD models.

Preparation of Constructs for Tran ctions and Microinjections

DNA clones for microinjection e cleaved with appropriate enzymes, such as *Sal*1, t1, etc., and the DNA fragments electrophoresed on 1 agarose gels in TBE buffer (Maniatis et al., 1989) The DNA bands are visualized by staining with ethidi bromide, excised, and placed in dialysis bags contai ng 0.3 M sodium acetate, pH 7.0. DNA is electroel ed into the dialysis bags, extracted with phen -chloroform (1:1), and precipitated by two volumes of hanol. The DNA

detail in Hogan et al. Manipulating he mouse embryo, Cold Spring Har or Laboratory, Cold pring harbor, NY (1986), the tea hings of which are corporated herein.

Transgenic Mice

Female mice six weeks of age a induced to superovulate wi a 5 IU injection .1 cc, ip) of pregnant mare serum gonadotropin (P G; Sigma) followed 48 hou s later by a 5 IU i ection (0.1 cc, ip) of human ch rionic gonadotropin hCG; Sigma). Females are pla ed with males immed tely after hCG injection. Twenty-one hours after h , the mated females are sacrificed by CO_2 asphyx tion or cervical dislocation and embryos are recover from excised oviducts and placed in Dulbecco's p sphate buffered saline with 0.5 bovine serum album (BSA; Sigma). Surrounding cumulus cells are remov with hyaluronidase (1 mg/ml). Pronuclea embryos are then washed and placed in Earle's balanc salt solution containing 0.5% BSA (EBSS) in a 37. C incubator with a humidified atmosphere at 5% CO_2, 9 air until the time of injection.

Randomly cycling adult female ce are paired with vasectomized males. Swiss Webs r or other comparable strains can be used for is purpose. Recipient females are mated at the ne time as donor females. At the time of embryo tran er, the recipient females are anesthetized with an in aperitoneal injection of 0.015 ml of 2.5% avert per gram of body weight. The oviducts are exposed by single midline dorsal incision. An incision is th made through the body wall directly over the oviduct The ovarian bursa is then torn with watchmakers orceps. Embryos to be transferred are placed in DPB and in the tip of a transfer pipet (about 10-12 em yos). The pipet tip is inserted into the infundibul and the embryos

<u>Embryon ... m (ES) Cell Metho ...</u>

<u>In ... uction of cDNA into ... S cells:</u>

Methods ... the culturing of E ... cells and the subsequent p ... ction of transgenic ... nimals, the introduction ... DNA into ES cells b ... a variety of methods such ... electroporation, ca ... ium phosphate/DNA precipitatio ... direct injection ... re described in detail in <u>Te ... ocarcinomas and embr ... nic stem cells, a practical ap ... ch</u>, ed. E.J. Robert ... n, (IRL Press 1987), the t ... ings of which are i ... orporated herein. Selection of ... desired clone of t ... nsgene-containing ES cells is ... plished through on ... of several means. In cases inv ... g random gene inte ... ation, an APP clone is co- ... cipitated with a gen ... encoding neomycin resistance. ... ansfection is carrie ... out by one of several metho ... described in detail ... n Lovell-Badge, in <u>Teratocar ... mas and embryonic s ... m cells, a practical ap ...</u>, ed. E.J. Robert ... n, (IRL Press 1987) or in ... ter et al <u>Proc. Natl Acad. Sci. USA</u> 81, 7161 (19 ... Calcium phosphate ... NA precipitation, direct injection, and electroporati ... are the preferred methods. In these proced ... es, 0.5 X 10^6 ES cells are plat ... into tissue cultur ... dishes and transfected ... a mixture of the l ... earized APP clone and 1 mg of p. V2neo DNA (Southern a ... Berg, <u>J. Mol. Appl. Gen</u>. 1 27-341 (1982)) precip ... tated in the presence of ... mg lipofectin in a f ... al volume of 100 µl. The cells are fed with selecti ... medium containing 10% fetal bovine serum i ... DMEM supplemented with G418 (b ... en 200 and 500 µg/m ... Colonies of cells resistant to G418 are isolate ... using cloning rings and exp nded. DNA is extract ... from drug resistant cl ... s and Southern blott ... ng experiments using an APP770 cDNA probe are used ... to identify those clones carrying the APP sequences. In some experiments, PCR methods are used t ... identify the clones of in ... rest.

Recipient fe..al s e mated such tha they will be at 2.5 to 3.5 d vs t-mating when req red for implantation it astocysts contai ng ES cells. At the time of ransfer, the rec ient females are anesthetized wi intraperitoneal njection of 0.015 ml of 2.5 rtin per gram of ody weight. The ovaries are xp se by making an inc ion in the body wall directl o he oviduct and t ovary and uterus are e ized. A hole is de in the uterine horn 25 gauge needle rough which the blastocysts e nsferred. After e transfer, the ovary and uteru pushed back int the body and the incision is lo ed y two sutures. is procedure is repeated on he site side if add ional transfers are to be ma e

<u>Identificati f ansgenic Mice a Rats</u>.

Tail sa 1-2 cm) are remov from three week old animals. D s prepared and a yzed by both Southern blot a CR to detect tra enic founder (F_0) animals and he r rogeny (F_1 and F_2)

<u>Patholo l udies</u>

The var $_0$, F_1, and F_2 anima that carry the microinjecte gene are sacrific by CO_2 asphyxiation a alyzed by immuno tology for neuritic plaques d neurofibrillar angles (NFTs) in the brain. Bra of mice and rats om each transgenic l . fixed in 4% par rmaldehyde and sectioned on ostat. Sections e stained with antibodies r e with the APP an r the ß peptide. Secondary an s conjugated wit fluorescein, rhodamine, h dish peroxidase, r alkaline phosphatase are ed to detect the imary antibody. These experime ermit identifica on of amyloid plaques and gionalization of ese plaques to specific are the brain. Plaqu ranging in size from 9 to 50 c aracteristically cur in the brains of AD patien n the cerebral cort , but also may be

The pr... fr..ctions are dena...ured in Laemmli sample buff..r ... electrophoresed ... SDS-Polyacrylam..e ...s. The proteins ...re be then transferred ...rocellulose filte... by electroblott... The filters are b...ocked, incubated with primary ... bo..ies, and finall... reacted with enzyme conju..a... secondary antibod...es. Subsequent incubation ..ith ..he appropriate chr...nogenic substrate reveals the po... ion of APP protei... .

Pathol... ic... and Behavioral St...dies

P... ...ical Studies

Immunoh... ...gy and thioflavi... S staining are conducted as d...scribed elsewhere he...ein.

In situ H...ridizations: Radio...ctive or enzymatical..y ...abeled probes are u...d to detect mRNA *in situ*. T... p...bes are degraded a...proximately to 100 nucleotides ...n ...gth for better pe...etration of cells. The procedu... ... ..hou et al. J. Ps...h. Res. 24,27-50 (1990) for f...x... and paraffin embe...ed samples is briefly desc...i...d below although si...ilar procedures can be empl...ye... with samples sectio...ed as frozen material. ...ara...fin slides for *in si...tu* hybridization are dewaxed ...n ...ene and rehydrat... in a graded series of et...an... and finally rin...d in phosphate buffered sal...n... (PBS). The sectio... are post-fixed in fresh 4% paraf...maldehyde. The sl...es are washed with PBS twice fo... ...minutes to remove ...raformaldehyde. Then the sec...io...s are permeabilize...by treatment with a 20 μg/ml ...ot...nase K solution. ...he sections are re-fixed in ...% p...aformaldehyde, a... basic molecules that could g...v... ...se to background ...robe binding are acetylated in a 0.1 M triethanolam...e, 0.3 M acetic anhydride solu...on for 10 minutes. The slides are washed in PB..., ..hen dehydrated in a graded series of ethanols and a...r dried. Sections a...e hybridized with antisense p...b..., ...sing sense probe...s a control. After approp...i...t... washing, bound r...ioactive probes

NIH3T3 or P... ...s were plated in... 100 mm dishes and transfected w...h a mixture of 5 ...g of the *SalI* fragment and 1 ...g of pSV2neo DNA (4... precipitated in the presence ... mg lipofectin (G...co, BRL) in a final volume ... μl. Polylysine ...oated plates were used for PC1... ...ls, which normally ...o not adhere well to tissue cu... ...e ...ishes. The cell... were fed with selection medi... ...ntaining 10% fet... bovine serum in DMEM or RPMI a... ...pplemented with ...18. Five hundred mg/ml (biol... ...weight) and 250 m.../ml of G418 were used to sele... co...nies form NIH3T3 ...nd PC12 cells, respectively ...fifteen days after t...ansfection, colonies of ...ls resistant to G418 ...ere isolated by cloning ring... ...a ...xpanded in T fla...ts. The presence of APP cDNA ... cells was detect...d by PCR using the procedure of ... and Faloona, Me...hods Enzymol. 155;335-350 ...7), the teachings o... which are incorporated herein.

Express...on o... APP in 25 coloni...s from each cell line was ana...ed by immunostaining (Majocha et al., 1988). Cel... w...r... grown to subconf...uence and fixed in a solution co...t...ing 4% paraformal...ehyde, 0.12 M NaCl, and 20 ... $...PO_4$, pH 7.0. The... were incubated overnight wi...h a primary monoclonal antibody against a synthetic ß ...ep...ide sequence (Maste...s et al., 1985) provided by ... Ronald Majocha, Mas...achusetts General Hospital, B...ton, MA, followed by a generalized anti-mouse antibody ...jugated to biotin (Jackson ImmunoResearc... ...s, PA). Immunost...ining was then performed by ...di...g avidin-horserad...sh peroxidase (HRP) (Vect... Labs, Burlingame, CA) and diaminobenzi...ine ...s the chromogen (...ajocha et al., 1985). The ...sults indicated that ...he pMTAPP-1 vector was expressi...g A... in both NIH3T3 a...d PC12 cells.

Example 2: **...pression of pEAPP-... in PC12 Cells.**

pEAPP-1 ... an example of the ... kb human APP gene promoter lin...e... to and controlling ...xpression of a

thus enco[illegible] ea[illegible] ins the leader seq[illegible]ence, followed by a shorte[illegible] r[illegible]ion of the ß peptide starting with the sequence [illegible]-val-gly-ser-of the [illegible] peptide followed by the 5 [illegible] amino acids of AP[illegible]. DNA from this construc[illegible] [illegible]fected into PC12 [illegible]ells as described above.

Example [illegible] Generation of Transge[illegible]ic Mice [illegible]xpressing APP under [illegible]he control of the [illegible]T-1 promoter.

Tra[illegible] [illegible]ce were made by mi[illegible]roinjecting pMTAPP1 [illegible] into pronucl[illegible]ar e[illegible]bryos. pMTAPP1 is an ex[illegible] the type of constru[illegible]t shown in Fig. 1a which [illegible] e[illegible]ably linked to the m[illegible]tallothionine promoter. T[illegible] procedures for microi[illegible]jection into mouse emb[illegible]yo [illegible]e described in "Mani[illegible]ulating the mouse embryo" [illegible] [illegible]gan et al. (1986). Only a brief descript[illegible]n [illegible] procedures is des[illegible]ribed below.

Mic[illegible] [illegible]tained from Taconic Laboratories (German [illegible] N[illegible]w York). Swiss Webs[illegible]ter female mice were use[illegible] f[illegible] e[illegible]bryo retrieval and i[illegible]plantation. B6D2F$_1$ ma[illegible]es [illegible]e used for mating an[illegible] vasectomized Swiss We[illegible]ste[illegible] [illegible]uds were used to si[illegible]ulate pseudopr[illegible]gna[illegible]y.

<u>Emb[illegible] [illegible]very</u>: Female mice, [illegible] to 8 weeks of age, were i[illegible] [illegible] to superovulate w[illegible]th 5 IU of pregnant [illegible] s serum gonadotropin [illegible]PMSG; Sigma) followed [illegible]8 [illegible]rs later by 5 IU of [illegible]uman chorionic gonadotr[illegible]pin [illegible]CG; Sigma). Females were placed with males im[illegible]edi[illegible]y after hCG injectio[illegible]n. Embryos were recovere[illegible] f[illegible] [illegible]xcised oviducts of [illegible]ated females 21 hours aft[illegible]r [illegible] in Dulbecco's phosph[illegible]ate buffered saline with [illegible] bovine serum album[illegible]n (BSA; Sigma). Surround[illegible]ng [illegible]mulus cells were remo[illegible]ed with hyaluron[illegible]das[illegible] [illegible]1 mg/ml). Pronuclea[illegible] embryos were then washed a[illegible]d p[illegible] ed in Earle's balanc[illegible]d salt solution contai[illegible]g [illegible] BSA (EBSS) in a 37. °C incubator with

infundi.u. ınd embryos were t ans:erred. After the transfe: incision was clos d b two sutures.

An. y ..f Mice For Trans.gene Integration: At three we.. age tail samples abo t 1 cm long were excised fo A analysis. The ail samples were digeste. L cubating with sh..ing overnight at 55°C in the pre ce of 0.7 ml 5 mM ris, pH 8.0, 100 mM EDTA, 0.5 S and 350 µg of pr tei ase K. The digeste m ial was extracted onc with an equal volume o nol and once with n e ual volume of phenol:ch orm (1:1 mixture). T e supernatants were mi.e h 70 µl 3 M sodiu ac tate (pH 6.0) and the DNAs w precipitated by a din equal volume of 100% etha The DNAs were sp n d wn in a microfuge, washed onc ith 70% ethanol, d ied and dissolved in 100 µl TE fer (10 mM tris pH 8.0 and 1 mM EDTA).

10-2 of DNAs were restrict d with BamH1, electroph d on 1% agarose g ls, blotted onto nitrocell paper, and hybri ize with ^{32}P-labeled APP cDNA ment. Transgenic nim ls were identified by autora raphy of the hybri ize nitrocellulose filters. DNAs were also an lyz d by PCR carried out by s ic primers to generat an 800 bp fragment.

A to f 671 pronuclear mbr os were microinje out of which 73 l ve nd 6 dead pups were born NA analysis identi ied 9 transgenic mice (5 females d 4 males) which re red to generate F_1 and F_2 tra nics. These anim ls c n be analyzed for expressio mRNA and protein of A P in different tissues a r analysis of behavio al and patholog abnormalities as d scr bed above.

4. a. al of claim 1 rod ced by introduct of he construct into n embryo, insertion the mbryo into a urr gate mother, and allowing mbryo to develop o t rm.

5. mammal of claim 1 wher in the mammal is a rodents.

6. ma al of claim 1 rod ced by mating transgeni mmals expressing diffe ent constructs.

7. mam al of claim 1 wher in the codon encoding val amino acid residue at 717 in the wild type APP e is mutated to encode n amino acid selected the group consisting of ile, phe, gly, tyr, leu, , pro, trp, met, s r, thr, asn, and gln.

8. mam al of any of claim 1 wherein the promoter he APP promoter of the same species of origin as mammal.

9. method for making a tran genic model for Alzheime isease comprising intr ducing into non-human mam n cells or embryo a construct selected from the p consisting of th AP770 cDNA; the APP770 cD bearing a mutation at amino acid 717; the APP751 cD A containing the KI protease inhibitor domain wi ut the OX2 domain in the construct; the APP751 cD bearing a mutation at amino acid 717; the APP695 cD the APP695 cDNA bearing a mutation at amino aci 7; the APP leader sequence followed by the ß pep de region plus the remaining carboxy terminal amino acids of APP; the APP leader sequence lowed by the ß peptide region plus the remaining rboxy terminal 56 amino acids with the addition mutation at amino aci 717; APP leader sequence owed by the ß peptide region; the ß peptide r gi n plus the remaining carboxy terminal 56 amino aci of APP; the ß peptide region plus the remaining rboxy terminal 56 amino acids of APP with the addit of a mutation at amino acid 717; a combinati enomic-cDNA APP gene construct; a

acid 71[illegible], [illegible]ably linked to a [illegible]rom[illegible]ter selected from the fol[illegible]: the human APP pr[illegible]mot[illegible]r, mouse APP promote[illegible], [illegible]APP promoter, met[illegible]llo[illegible]hionine promoter, rat neuron [illegible]ific enolase pro[illegible]te[illegible], human ß actin gene pr[illegible]mo[illegible] human platelet d[illegible]riv[illegible]d growth factor B (PDGF-B[illegible] gene promoter, r[illegible]t s[illegible]dium channel gene promoter, [illegible] myelin basic pr[illegible]tei[illegible] gene promoter, human cop[illegible]nc superoxide dis[illegible]mut[illegible]se gene promoter, and mamma[illegible]OU-domain regulatory gene promoter; transge[illegible]ic [illegible]ls or animal ce[illegible]ls [illegible]ontaining a construct s[illegible]e[illegible]ted from the gro[illegible]p c[illegible]nsisting of a human y[illegible]as[illegible] [illegible]ificial chromoso[illegible]e c[illegible]nstruct controlled by the hum[illegible]n [illegible]P promoter and a hum[illegible]n yeast artificial chromosom[illegible] [illegible]struct controlled by [illegible]he human APP promoter [illegible]ne addition of a [illegible]mut[illegible]tion at amino acid 717, and [illegible] [illegible]nic animals or [illegible]nim[illegible]l cells containing an APP gen[illegible] [illegible]e[illegible]ected from the g[illegible]oup consisting of the endogenous [illegible]e or rat APP gen[illegible] mo[illegible]ified such that sequences [illegible]e resident chrom[illegible]so[illegible]l APP gene beyond the recom[illegible]tion point in APP [illegible]xon 9 are replaced by the analo[illegible] [illegible]uman sequences and t[illegible]e analogous human sequences [illegible] the addition of [illegible] mu[illegible]ation at amino acid 717; a[illegible]d [illegible]ombinations ther[illegible]of, to the compound to be tested [illegible] [illegible]etermining if th[illegible]re [illegible]s altered expressio[illegible] [illegible] APP.

12. [illegible] method of claim 1[illegible] us[illegible]ng transgenic mammals f[illegible]r comprising dete[illegible]mi[illegible]ing if there is altered b[illegible]r of the transge[illegible]ic [illegible]ammals after administr[illegible]t[illegible] of the compound [illegible]o t[illegible]e animal.

13. [illegible]ethod of claim 1[illegible] w[illegible]erein the mammals are rodent[illegible].

FIG. 4a

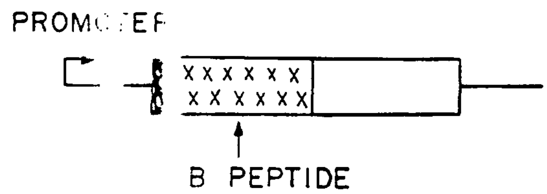


FIG. 4b

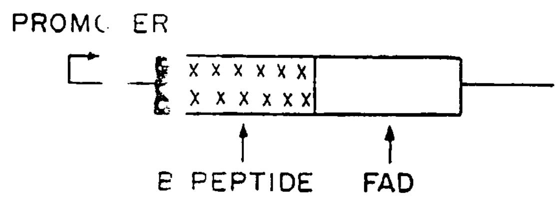


FIG. 5

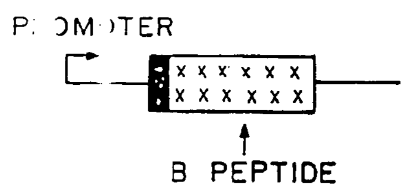


FIG. 6a

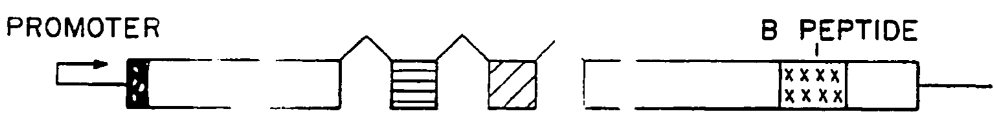
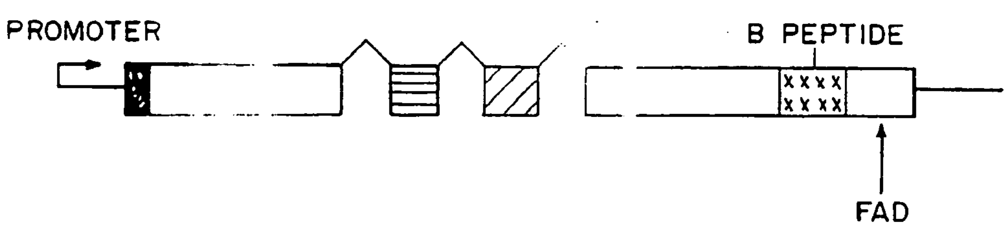


FIG. 6b



INTE[illegible] SEARCH REPORT

ternational application No.
PCT/US 92/11276

A. CLASSIFICATIO[illegible] MATTER

IPC5: C12N 15/[illegible] 027, C12N 5/10, C07K 15/00

According to Internatio[illegible] ation (IPC) or to both national classification and IPC

B. FIELDS SEARCH[illegible]

Minimum documentation [illegible] hed [illegible] fication system followed by classification symbols)

IPC5: C12N, C07[illegible]

Documentation searched o[illegible] num documentation to the extent that such documents are included in the fields searched

Electronic data base cons[illegible] ernational search (name of data base and, where practicable, search terms used)

C. DOCUMENTS C[illegible] TO BE RELEVANT

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X	WO, A1, [illegible] (CALIFORNIA BIOTECHNOLOGY INC.), 26 [illegible] 1991 (26.12.91), figures 4-7, claims 1-4 a[illegible] -13, 15-16, 29-33, 61 and 64-65, exa[illegible] es [illegible], page 60, line 10 - page 61	1-6,9-13
Y		1-13
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Y	Nature, [illegible] lume 352, July 1991, D. Quon et al., "For[illegible] f beta-amyloid protein deposits in brai[illegible] nsgenic mice", page 239 - page 241, page [illegible] 39, [illegible] ge 241 left column	1-13
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[X] Further documents [illegible] list[illegible] the continuation of Box C. [X] See patent family annex.

* Special categories of cite[illegible]:

"A" document defining the ge[illegible] state [illegible] art which is not considered to be of particular relev[illegible]

"E" erlier document but pub[illegible] on [illegible] the international filing date

"L" document which may th[illegible] oubt[illegible] ority claim(s) or which is cited to establish the pub[illegible] on [illegible] another citation or other special reason (as specifi[illegible])

"O" document referring to a[illegible] dis[illegible] e, use, exhibition or other means

"P" document published prior [illegible] ational filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual comp[illegible] of [illegible] international search	Date of mailing of the international search report
19 April 1993	13. 05. 93
Name and mailing addre[illegible] the [illegible] European Patent Offic[illegible] 5818 Patentlaan 2 NL-2280 HV [illegible] vijk Tel. (+31-70) 34[illegible] 040 Tx. 31 651 epo nl, Fax: (+31-70) [illegible] 30[illegible]	Authorized officer Carl Olof Gustafsson

Form PCT/ISA/210 (second s[illegible]) (J[illegible] 1992)

INTE... ...N... SEARCH REPORT

International application No.
PCT/US 92/11276

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The EM... ...nal Volume 10, No 2, 1991, D. ... ...ak ... al., "Regulatory region of human am... precursor protein (APP) gene promotes neuro...-specific gene expression in the CNS of trans... ...ic mice", page 289 - page 296, figure 2, Discu... ...on --	1-13
Y	Nature, ..., February 1991, A. Goate et al. "S...greg... ...ion of a missense mutation in the amyl... precursor protein gene with familial Alzhei...er's disease" page 704 - page 706 --	1,7,9,11
Y	Nature, V... ...me ..., October 1991, M-C. ... ...tie... ...arlin et al., "Early-onset alzheimer's disease caused by mutations at codon 717 ... the beta-amyloid precursor protein gene" pag... ...44 - page 846 --	1,7,9,11
Y	Science, ...ume 2...4, October 1991, J. Murrell et al., "... Mutation in the Amyloid Precursor Protein Associ... ...ed ... ...h Hereditary Alzheimer's Disease" --	1,7,9,11
P,X	WO, A, 92... ...187 (THE UPJOHN COMPANY), 16 April 1992 (16.0... ...2), page 8, line 15 - line 33, examples 1 and 2 --	1-6,8-13
P,X	WO, A, 921... ...9 (I... ...PERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE), 6 August 1992 (06.08.92), claims, examples 3 and 4, pages 10, 11, 13, 14 and 19-21 --	1-13
E,X	WO, A1, 9...189 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 4 February 1993 (04.02.93), claims, example -- --------	1-5,9,11-13

S, .9144

INTERN... ...NAL SEARCH REPORT
Inform... on patent family members

31/03/93

International application No.
PCT/US 92/11276

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 911...1	2./12/91	AU-A- 8210291	07/01/92
WO-A1- 89066..9	27/07/89	AU-A- 3056289	11/08/89
EP-A1- 04517...	16/10/91	NONE	
WO-A- 920618.7	16/04/92	AU-A- 8768191	28/04/92
WO-A- 92130...	06/08/92	AU-A- 1169492	27/08/92
WO-A1- 93021...	04/02/93	NONE	